Meet Our Uncle: 12 Stability Applications on One Platform

Uncle is an all-in-one stability platform that enables twelve different applications with one instrument. Fluorescence, static light scattering (SLS) and dynamic light scattering (DLS) detection methods are used to characterize protein stability (Figure 1). Multiple detection methods means that multiple measurements, such as thermal melting, aggregation, and sizing are possible with the same set of samples. Using light scattering and fluorescence together is needed to get the whole story, so fluorescence can see unfolding even when aggregation obscures protein size changes. Temperature control (15–95 °C) and sealed samples provide greater flexibility in how that characterization can be performed. Samples are loaded into Unis, low volume, multi-well quartz cuvette chambers. And since you can run low volumes of 48 samples at a time, you can thoroughly characterize more biologics and formulations earlier than before.

Figure 1: Uncle: a one-stop stability platform.

This technical note describes how you can use Uncle for many of your key stability measurements.

1. Thermal melting (T_m)

Determine the melting temperature of your protein by directly measuring unfolding through intrinsic fluorescence. As your protein unfolds and amino acids rearrange, changes in the fluorescence behavior of tryptophan and tyrosine signal conformational changes of your protein. Intrinsic fluorescence is one of the only direct ways to observe the unfolding of a protein, and can do so even after aggregation begins, unlike methods based on light scattering or calorimetry. Rank the stability of your constructs or compare different formulations to find the most favorable conditions. In most cases there is a change in fluorescence intensity or a peak shift as proteins unfold. There are many exceptions to this, and better ways to analyze the data. Uncle measures the full fluorescence spectrum to ensure that you have all the data you need, analyzed in all the ways that you need.

2. Thermal aggregation (T_{agg})

Find out how and when your protein aggregates during a thermal ramp, by using SLS at two different wavelengths at the same time to detect both small and larger particles. Rank your constructs or formulations, or find excipients that prevent or delay the onset of aggregation. Since proteins can unfold and can aggregate, measuring T_m concurrently with T_{agg} easily determines when an unfolding event leads to aggregation. Using fluorescence, which doesn't rely on size changes, makes it possible to measure T_m even after aggregation has already begun.

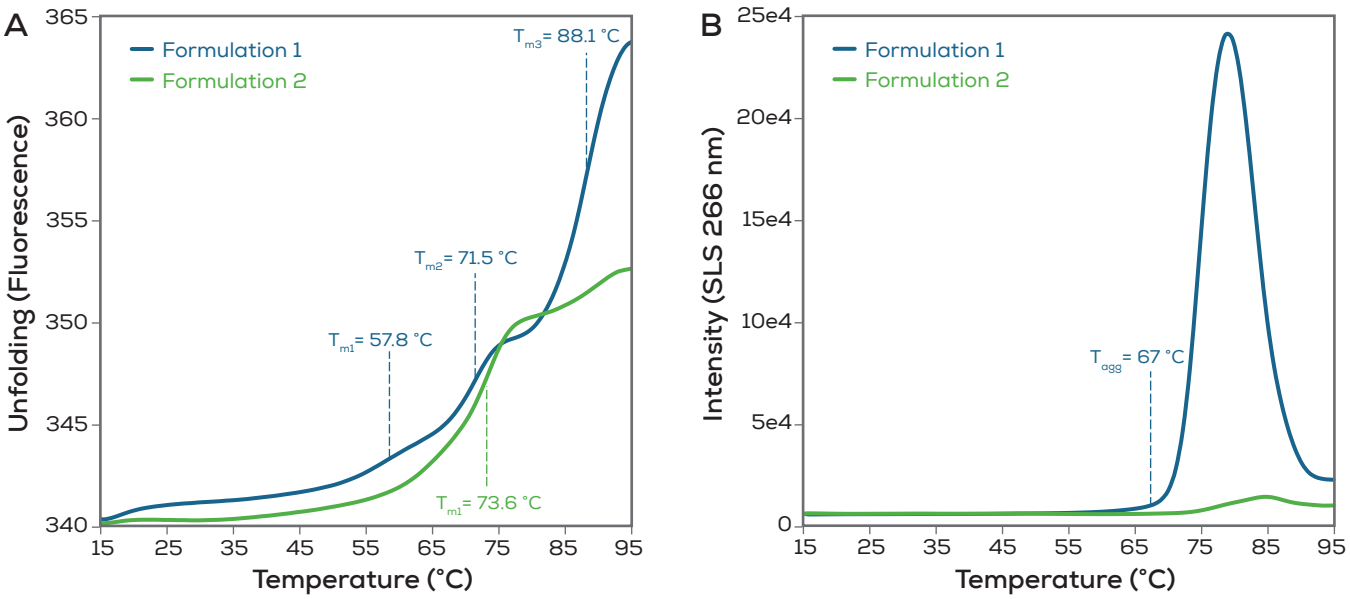

Figure 2: Thermal melting (**A**) and thermal aggregation (**B**) profiles of an antibody in two different formulations.

Combined T_m and T_{agg} method: A monoclonal antibody was prepared in multiple formulations at 0.5 mg/mL. Nine µL of each sample was loaded into a Uni and run with a thermal ramp from 15-95 °C, with a ramp rate of 0.3 °C/minute. The BCM (barycentric mean) of the fluorescence intensity was selected as the best analysis method.

T_m results: In Formulation 1, the antibody undergoes three distinct transitions, determined by the software to occur at 57.8 °C, 71.5 °C, and 88.1 °C (Figure 2A). The same antibody in Formulation 2 shows improved thermal stability, as the protein does not undergo a transition until later, with a T_m of 73.6 °C.

T_{agg} results: SLS at 266 nm (Figure 2B) shows that the antibody in Formulation 1 undergoes a sudden onset of aggregation at 67 °C, which is after the first unfolding transition. At about 78 °C, the intensity at 266 nm reaches a maximum and the aggregated protein begins to precipitate. In contrast, the same antibody in Formulation 2 shows almost no aggregation throughout the thermal ramp. SLS performed simultaneously at 473 nm (not shown) measures a similar result.

Even though the protein began to aggregate at 67 °C, T_m2 and T_m3 at higher temperatures were still detectable via intrinsic fluorescence.

3. Thermal melting with SYPRO

Differential scanning fluorimetry (DSF) is the classic higher throughput method to determine melting temperatures. Your protein is combined with an extrinsic dye like SYPRO® Orange, which undergoes a thermal shift when bound to hydrophobic surfaces of unfolded protein. This application can be useful for instances where there are few or no tryptophan residues in the protein, or assessing protein stability at very low concentrations. As with all other applications on Uncle, the software streamlines your data analysis to save time.

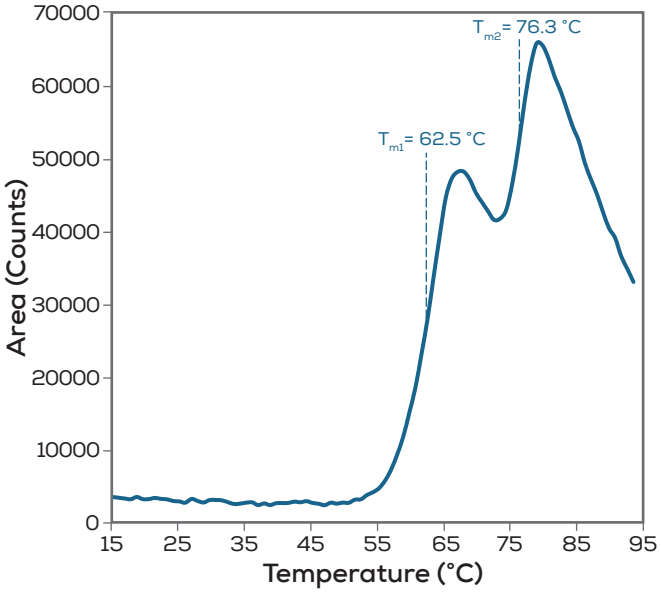

Figure 3: Thermal melting of an antibody in the presence of SYPRO® Orange dye.

Method: A human monoclonal antibody was formulated at 1 mg/mL with SYPRO® Orange. Nine µL was loaded into a Uni and run with a thermal ramp from 15 °C to 95 °C, with a ramp rate of 0.5 °C/minute and excitation at 473 nm. Uncle software used the area under the curve between 510-680 nm to analyze the data and calculate the inflection points of the transition curve.

Results: The antibody undergoes two distinct transitions, calculated by the software to occur at 62.5 °C and 76.3 °C (Figure 3). The intensity of the fluorescent signal from the SYPRO® dye is low when the protein is folded, as it is quenched by the aqueous environment of the buffer. The signal increases dramatically upon exposure of hydrophobic regions as the protein unfolds at higher temperatures, and then decreases again upon dissociation and aggregation of denatured protein.

4. ΔG

Forced degradation is an established surrogate for room-temperature stability assessments, but thermal ramp methods do not always distinguish conditions of similar stability. For those tough nuts to crack, ΔG values, which provide a quantitative measurement of protein stability, can stratify conditions by measuring under an orthogonal stress condition. By exposing a protein to a chemical denaturant and measuring the resulting change in fluorescence, you can calculate the energy required to unfold it.

Method: A 32-point denaturation curve of urea from 2 to 9.2 M was made with a final concentration of 864 µg/mL of a therapeutic antibody. Samples were incubated overnight at room tem-

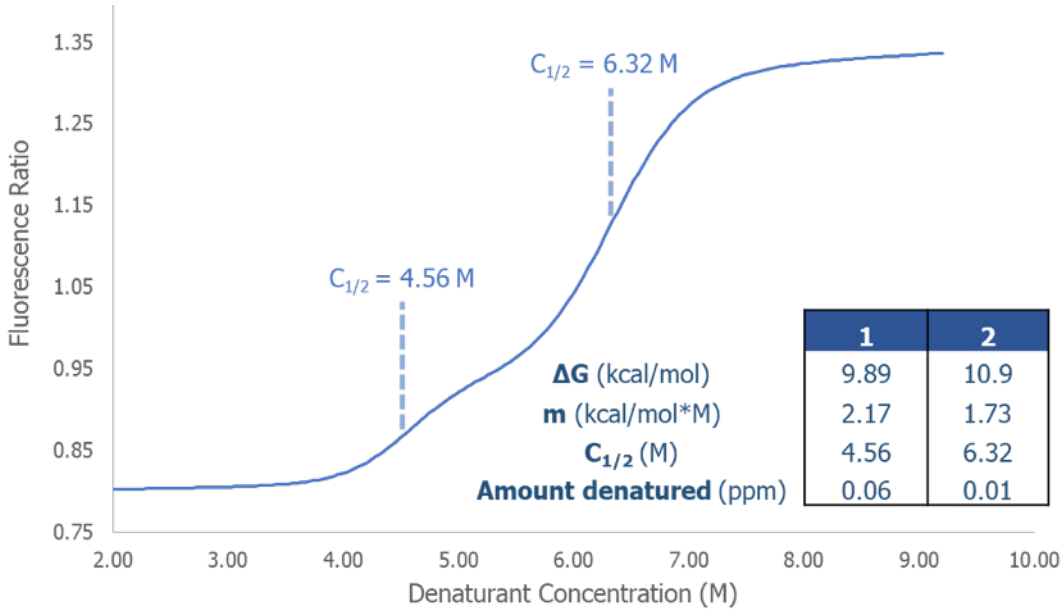

Figure 4: Denaturation curve for a therapeutic antibody. Values in the table were calculated by Uncle software using a 3-state model.

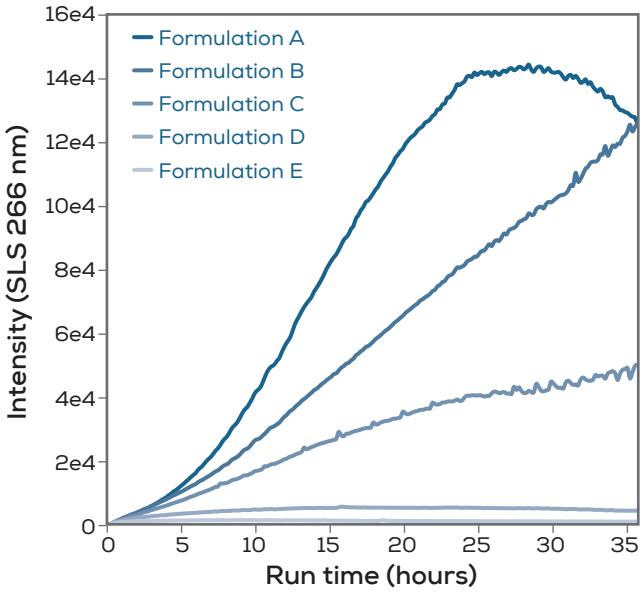

Figure 5: Isothermal aggregation of an antibody in five different formulations.

perature to ensure that equilibrium was reached. Nine µL of each was loaded into Unis and read at 25 °C.

Results: Figure 4 shows the ratio of fluorescence at 350/330 nm for the antibody as a function of denaturant concentration. The protein undergoes two distinct transitions at denaturant concentrations of 4.6M and 6.3M. Uncle Analysis software fit the data using a 3-state model and calculated ΔG values of 9.89 kcal/mol and 10.9 kcal/mol for the two transitions, respectively. At equilibrium and room temperature, there was 0.06 ppm denatured protein for the first transition and 0.01 ppm denatured protein for the second transition in the sample.

5. Isothermal stability

Isothermal stability is another approach to stability assessment. Rather than ramping temperature to cause a melting transition, samples are held for a longer time at temperatures just under the expected T_m. Samples are sealed in the Uni cuvettes, and a temperature can be held for hours or days without evaporating. Choose simultaneous fluorescence and SLS to monitor unfolding and aggregation of your samples over time, or go with DLS if you'd like to get an idea of how the size of molecules or aggregates changes over time.

Method: A human antibody was formulated at 1 mg/mL in PBS (Formulation A) or with increasing amounts of arginine (Formulations B–E). Nine µL of each sample was loaded into a Uni and held at 60 °C for 36 hours, below the melting temperature of the antibody.

Results: The graph (Figure 5) shows the light scattering intensity at 266 nm. Aggregation decreases with increasing concentrations of arginine, and the magnitude of the differences is more pronounced at longer incubation times.

6. Thermal recovery

Check on the reversibility of unfolding events, or monitor how temperature shifts affect your molecules. Pick a temperature (or several), and ramp up and down to see whether your protein can refold in your formulations of choice. Choose simultaneous fluorescence and SLS to monitor unfolding, refolding, and aggregation, or choose to collect DLS data if you'd like to monitor the conformational state of your protein by following the hydrodynamic size over time.

Method: A human antibody was prepared in PBS at 5 mg/mL. Nine µL of sample was loaded into a Uni and run with three temperature ramps from 15 °C to 58 °C, with 20 minute holds at each temperature, followed by three temperature ramps from 15 °C to 70 °C, with 20 minute holds at each temperature.

Results: The graph shows the unfolding and refolding of the protein, as measured by BCM of the fluorescence signal, as well as the light scattering intensity at 473 nm (Figure 6). When the protein is ramped to a temperature below its T_m the small amount of unfolding seen is partially reversible, and does not result in any aggregate formation. When the protein is ramped up to 70 °C, however, larger particles form irreversibly and the intrinsic fluorescence signal remains high even when the samples are cooled back to 15 °C.

7. Sizing and Polydispersity

DLS is a highly sensitive tool for measuring the hydrodynamic size of proteins and small molecules in

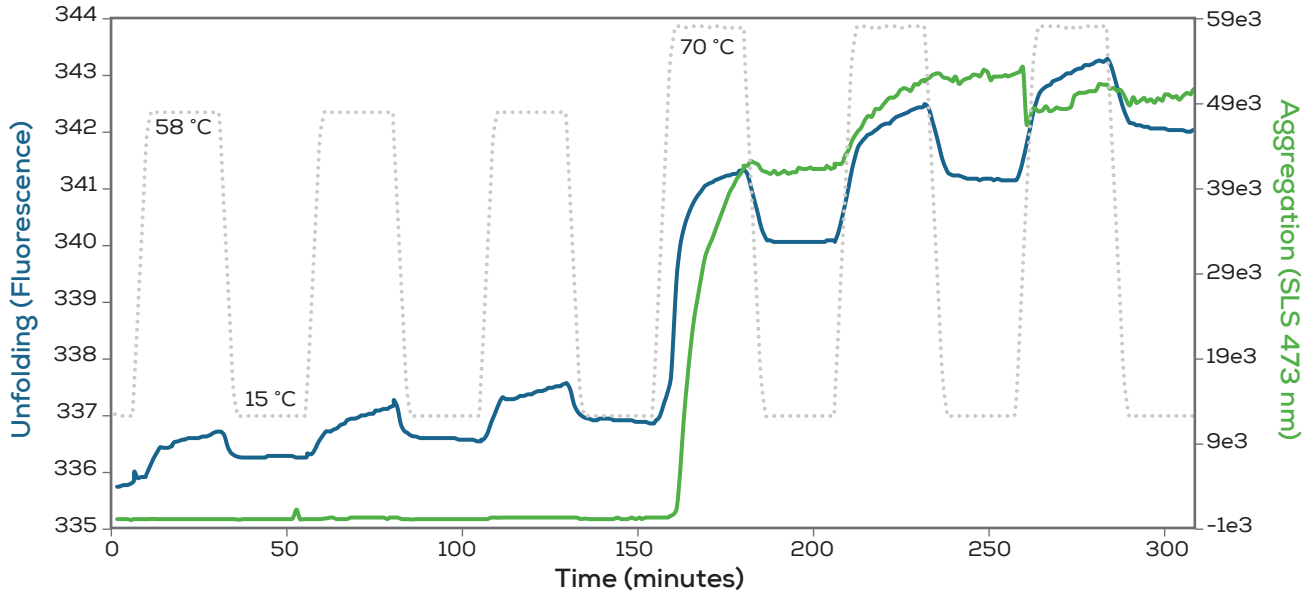

Figure 6: Thermal recovery of a protein over six rounds of heating and cooling.

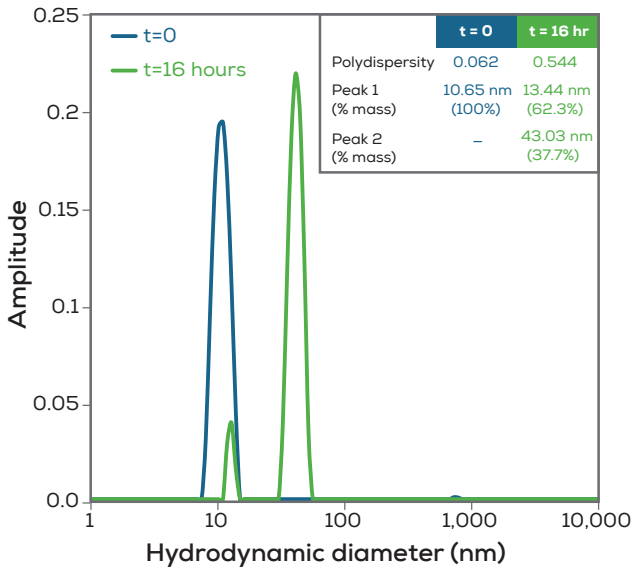

Figure 7: Size and polydispersity of an antibody at the beginning and end of an isothermal experiment.

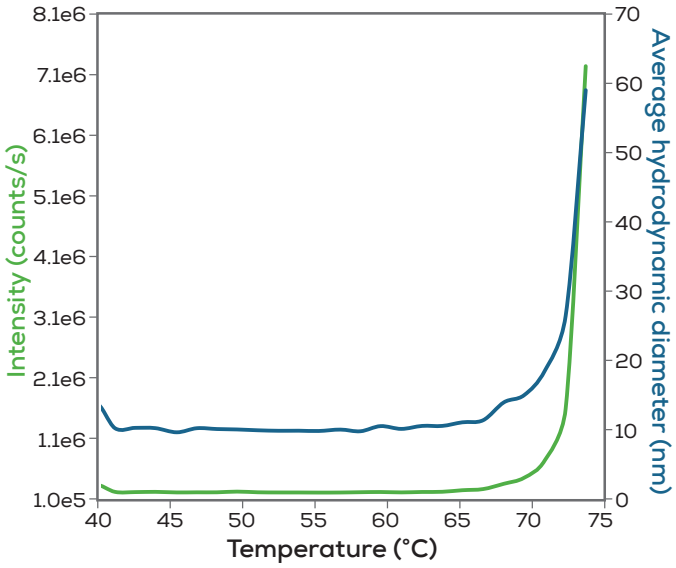

Figure 8: Average particle size and light scattering intensity of an antibody over a thermal ramp.

solution. The sensitivity of Uncle meets the highest industry standard, and you can size proteins at concentrations as low as 0.05 mg/mL. Use DLS to identify the presence of aggregates or impurities, and confirm that your molecule is the size you expect it to be. Temperature control enables you to get sizing information at any temperature between 15–95 °C.

Polydispersity is a measurement of non-uniformity in your sample. If particles are not uniform in size, a higher polydispersity will be measured. A low polydispersity value indicates more uniformity in the size of the particle. Without using any additional protein or adding any set-up time, get more information on polydispersity, and follow it up with other experiments on the same samples.

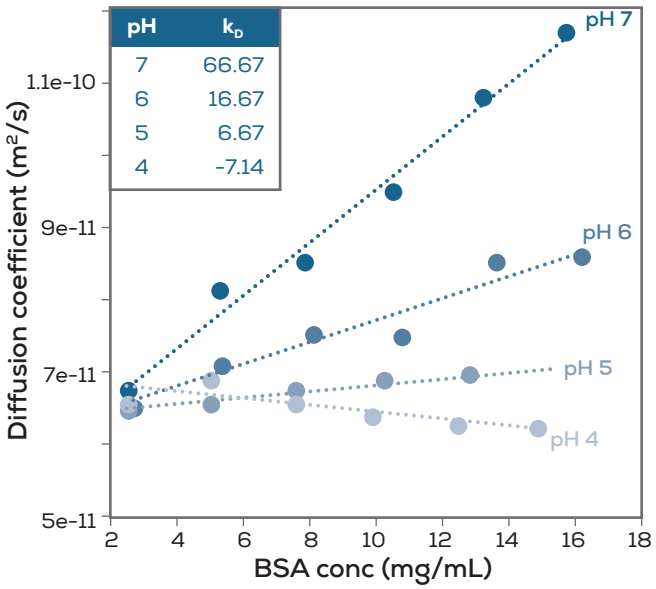

Figure 9: Diffusion coefficient as a function of protein concentration over pH 4-7, and the calculated k_D values for each condition.

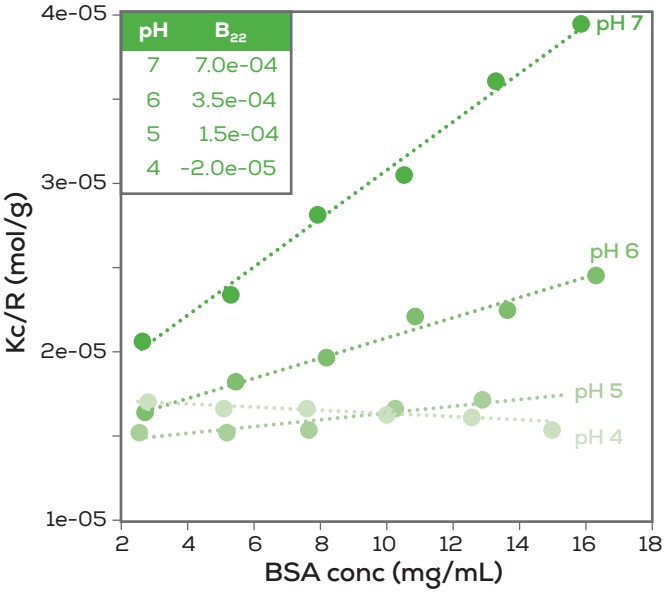

Figure 10: Scattering intensity as a function of BSA concentration over pH 4–7, and the calculated B_{22} values for each condition.

Method: Nine µL of a monoclonal antibody at 0.5 mg/mL was loaded into a Uni and held at 16 hours at 62 °C, with 4 DLS acquisitions of 5 seconds each, measured every 10 minutes. The size distribution of the sample at the beginning (t=0) and the end (t=16 hours) is shown in the graph (Figure 7).

Results: At the beginning of the run, the antibody has one peak at the expected size, and a polydispersity value below 0.1, indicating a monodisperse sample. After 16 hours at 62 °C, below the T_m or T_{agg} for this formulation, there are two distinct (larger) peaks, and the polydispersity has increased to above 0.5, indicating significant aggregate content.

8. Sizing with thermal ramp

A different way to determine or confirm the thermal stability of your biologic is to measure the hydrodynamic size of your proteins with DLS as they undergo conformational changes under thermal stress. An increase in size as measured by DLS can be evidence of unfolding. However, after aggregation begins, a true melting temperature cannot be determined with light scattering alone. Uncle's multiple detection modes allow you to get a clearer picture of unfolding and aggregation, and confidently monitor conformational changes in your proteins.

Method: Nine µL of a monoclonal antibody at 0.5 mg/mL was loaded into a Uni and run with a thermal ramp from 40-74 °C, with a ramp rate of 0.3 °C/minute. Each measurement was made with two DLS acquisitions of 3 seconds each.

Results: Both the overall mean particle size and the intensity of the scattered light remain fairly constant until about 70 °C, when both measurements show a dramatic increase (Figure 8). That corresponds with the second major unfolding transition of the antibody in this formulation.

9. k_D: (Diffusion Interaction Parameter)

Determine the diffusion interaction parameter of your proteins in different formulations, to help predict their colloidal stability or likelihood of aggregation. After you narrow down to better constructs or formulations, verify your favorite conditions with this non-invasive technique. You can also get B_{22} values at the same time, with one experimental set-up and just minutes of time.

Method: Bovine Serum Albumin (BSA) was prepared at 15 mg/mL in buffers (sodium acetate, histidine, or phosphate) at each indicated pH. The exact concentration was verified by absorbance, and 6 dilutions were made at each pH, down to 2 mg/mL. Nine µL of each sample were loaded in triplicate in a Uni, and run with 4 DLS acquisitions of 5 seconds each. Uncle software uses the hydrodynamic diameter measured for each sample, in conjunction with information about the solvent, to calculate the diffusion coefficients. The slopes and intercepts of the lines are then used to calculate k_D values.

Results: A strong positive value for k_D (as seen at pH 7 for this protein), suggests net repulsive interactions (Figure 9). The k_D values decrease with decreasing pH. At pH 4, the negative k_D value indicates net attractive interactions between molecules. This is in agreement with previous observations of BSA under these conditions.

10. B_{22}: (Second Virial Coefficient)

The second virial coefficient, or self-interaction parameter, is an established and valuable way to study pairwise interactions between protein molecules in your sample. At the same time that you measure k_D, and with the exact same sample, get an independently-calculated measurement for confirmation and use it to help predict the aggregation propensity of a protein in your favorite formulations. A positive B_{22} value indicates net repulsive forces between protein molecules, while a negative B_{22} value indicates that the protein might be prone to self-association.

Method: Toluene scattering intensity was measured in a Uni to calibrate the standard parameters for the instrument. BSA was prepared at 15 mg/mL in buffers (sodium acetate, histidine, or phosphate) at each indicated pH. The exact concentration was verified by absorbance, and 6 dilutions were made at each pH, down to 2 mg/mL. Nine µL of each sample were loaded in triplicate in a Uni, and run with 4 DLS acquisitions of 5 seconds each. Uncle software uses the light scattering intensity from each sample to calculate the Kc/R values, and

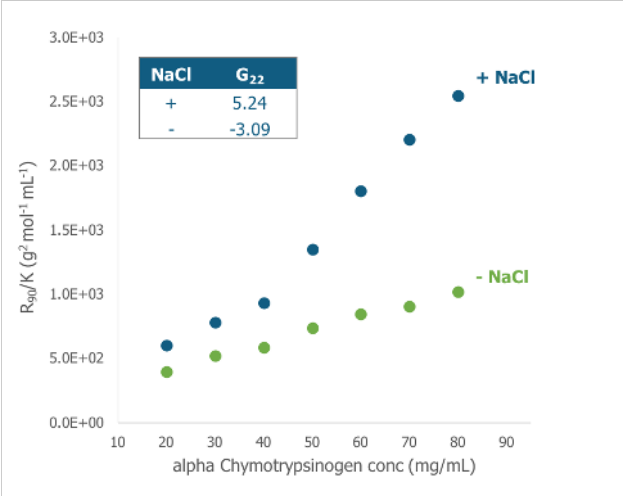

Figure 11: Scattering intensity as a function of protein concentration at pH 5, and the calculated G_{22} values for the highest protein concentration (80 mg/mL).

the slopes of the lines fit to the data were used to calculate B_{22}.

Results: A strong positive value for B_{22} (as seen at pH 7 for this protein), suggests net repulsive interactions, which is in agreement with the independently measured k_D value for the same condition (Figure 10). Likewise, at pH 4, the negative B_{22} value indicates attractive interactions between molecules, leading to the conclusion that it is not a favorable formulation for this protein.

11. G_{22} : (Kirkwood-Buff Integral)

G_{22} is a more rigorous way to measure the net self-interaction parameter of protein molecules, especially at high concentrations, by accounting for protein-solvent and protein-solute interactions in your sample. Unlike B_{22}, a negative G_{22} value indicates net repulsive forces between protein molecules, while a positive G_{22} indicates possible self-association. To save valuable sample and time, you can re-analyze the same B_{22} dataset to get an independently-calculated G_{22} value for high protein concentrations.

Method: Toluene scattering intensity was measured in a Uni to calibrate the standard parameters for the instrument. Alpha-chymotrypsinogen was prepared at 80 mg/mL in 40 mM sodium acetate buffer (with or without 300 M NaCl) at

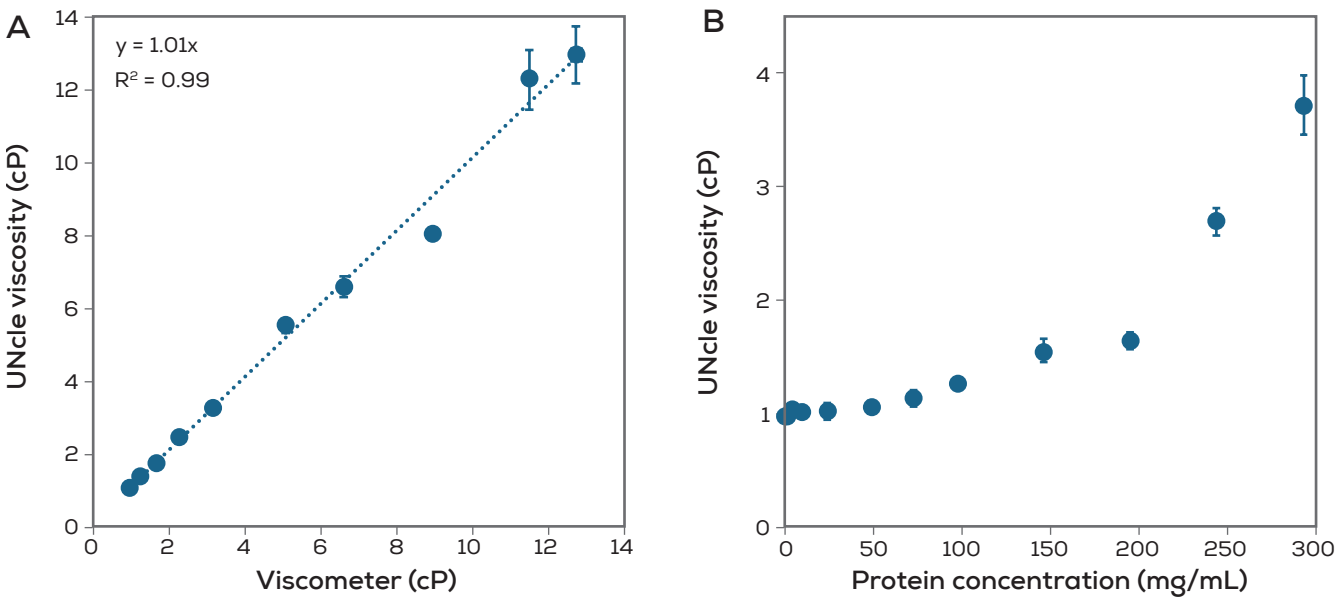

Figure 12: Correlation of glycerol standards measured on Uncle and a viscometer (**A**), and the viscosity of a protein at increasing concentration as measured by Uncle (**B**).

pH 5. The exact concentration was verified by absorbance, and 6 dilutions were made down to 20 mg/mL. Nine µL of each sample were loaded in triplicate in a Uni, and run with 4 DLS acquisitions of 5 seconds each. Uncle software uses the light scattering intensity from each sample to calculate the R_{90}/K values, which are used in conjunction with the expected molecular weight of the protein to calculate G_{22} for each protein concentration.

Results: A strong upward trend for R_{90}/K (as seen with the 300 M NaCl formulation of this protein) suggests net attractive interactions, which is in agreement with the calculated positive G_{22} value at the highest protein concentration for the same condition (Figure 11). The protein formulation without salt shows a modest increase in R_{90}/K along with a negative G_{22} value at the highest protein concentration, indicating that the protein does not have a propensity to self-associate under these conditions.

12. Viscosity

Compare the viscosity of different proteins, or find out how formulation ingredients or excipients increase or reduce the viscosity of your solutions much earlier in the development process. Uncle uses smaller volumes of sample than a traditional viscometer, so you can screen through more conditions with much less protein.

Method: For glycerol standards, 1 µL of 100 nm polystyrene beads and 1 µL of a Tween solution were mixed with 100 µL each of 10 glycerol solutions ranging from 0 to 67% (w/w). For protein samples, BSA was prepared in PBS at concentrations ranging from 1–300 mg/mL, and each sample was mixed with 100 nm polystyrene beads and dilute Tween. Samples containing no protein were included for reference bead size measurements. Nine µL of each sample were loaded into a Uni and run with 4 acquisitions of 5 seconds each at 23 °C. For glycerol standards, data was correlated with viscosity measurements collected on a Rheosense microVISC, using samples with PBS substituting for the beads.

Glycerol results: There is excellent correlation between glycerol solutions measured on Uncle and on a viscometer to viscosities as high as 14 cP (Figure 12A). For each instrument, triplicate reads were averaged to obtain the value on the graph, but Uncle requires only 9 µL for each measurement while the viscometer required 400 µL of sample for the three measurements.

Protein results: The viscosity of solutions of BSA as high as 300 mg/mL were measured in Uncle, yielding reproducible viscosity values of up to 4 cP

(Figure 12B). For Newtonian fluids such as these, the values correspond well with previously reported values obtained by other methods.

Summary

Uncle combines three detection methods to enable more stability measurements on a single platform. This provides more flexibility to researchers by enabling combinations of measurements to be performed in tandem; for example, follow up sizing and polydispersity measurements with thermal melting and aggregation. In other instances, values can be obtained simultaneously (such as k_D and B_{22}). In all, twelve different applications can be performed with one instrument, greatly reducing the protein sample volume requirements and the necessity to determine stability with multiple single-measurement instruments.

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